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BASIC—LIVER, PANCREAS, AND BILIARY TRACT

Ras Activity Levels Control the Development of Pancreatic Diseases

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BACKGROUND & AIMS: Differentiated pancreatic acinar cells expressing endogenous levels of mutant K-Ras do not spontaneously develop pancreatic ductal adenocarcinoma (PDAC). However, we hypothesized that acinar cells would develop PDAC in the presence of Ras activity levels mimicking those of human tumor cells. **METHODS:** We measured Ras activity in PDAC cells from mice and humans using a Raf pull-down assay. We compared the effects of acinar cell expression of mutant K-Ras at endogenous and elevated levels on Ras activity and on the development of PDAC. **RESULTS:** Ras activity was greatly elevated in PDAC cells compared with nontransformed cells expressing endogenous levels of mutant K-Ras. Expression of endogenous levels of mutant K-Ras in differentiated acinar cells resulted in moderately elevated Ras activity and in sparse murine pancreatic intraepithelial neoplasias (mPanINs) that did not spontaneously advance to PDAC unless the tumor suppressor p53 was simultaneously deleted. In contrast, expression of mutant K-Ras at higher levels generated Ras activity equal to that in PDAC. High Ras activity mimicking levels in PDAC led to acinar cell senescence and generated inflammation and fibrosis resembling the histologic features of chronic pancreatitis. With higher Ras activity in acinar cells, abundant mPanINs formed and spontaneously progressed to both cystic papillary carcinoma and metastatic PDAC. **CONCLUSIONS: There is an important relationship between Ras activity levels and the progression of PDAC. Sufficient Ras activity in pancreatic acinar induces several important pancreatic disease manifestations not previously reported and supports a potential direct linkage between chronic pancreatitis, cystic papillary carcinoma, and PDAC.**

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the United States.[1] Currently, there are no effective nonsurgical therapies for this disease. Therefore, considerable efforts have been made to develop animal models of PDAC useful for developing and testing new treatments. Recently, mouse models have been described that faithfully recapitulate many important aspects of the human disease.[2–4] These mouse models were developed by expression of activated mutants of K-Ras at endogenous levels from its native promoter in embryonic cells. These animals develop precursor lesions, mouse pancreatic intraepithelial neoplasias (mPanINs),[2] which progress over the course of 1–2 years into PDAC resembling the human disease. These models confirmed the potential role of K-Ras mutations as an initiator of PDAC and validated a model of PDAC progression starting from PanIN lesions and progressing to metastatic disease.[2,5]

It is well known that activating Ras mutations can induce either proliferation or senescence depending on the activity levels. In the mammary gland, chronic low-level Ras induction results in tumor formation only after the spontaneous up-regulation of activated Ras and evasion of senescence checkpoints.[6] In the pancreas of mice bearing targeted activating mutations at the endogenous K-Ras locus, a minor subset of cells display hyperproliferation and tumorigenesis.[2,4] In these models, the levels of Ras activity were significantly elevated in tumor lysates compared with nontumor tissue expressing mutant K-Ras.[7] These findings suggested that increased Ras activity may also play a crucial role in the process of pancreatic tumorigenesis.

In this study, we examined the effects of elevating Ras activity to different levels in pancreatic acinar cells in genetically engineered mice. We found that pancreatic

Abbreviations used in this paper: CP, chronic pancreatitis; CPC, cystic papillary carcinoma; GFP, green fluorescent protein; mPanIN, murine pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma; TGF, transforming growth factor; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

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acinar cells were transformed by endogenous levels of mutant K-Ras only in combination with genetic ablation of the tumor suppressor p53. Interestingly, Ras activity was greatly up-regulated in all PDAC cells examined, whether mouse or human. Expression of high levels of mutant K-Ras in acinar cells using a transgenic approach led to levels of Ras activity similar to those found in PDAC cells and caused mice to develop abundant mPanINs, cystic papillary carcinoma (CPC), and PDAC. High levels of Ras activity also led to widespread senescence of acinar cells and the development of profound inflammation and fibrosis resembling the histologic features of human chronic pancreatitis (CP). Thus, a novel model expressing elevated levels of mutant K-Ras in pancreatic acinar cells developed CP, CPC, and PDAC, providing the first evidence of a potential direct link between these diseases.

Materials and Methods

Detailed materials and methods are described in the Supplementary Materials and Methods.

Genetically Engineered Mice

cLGL-KRas^{G12V} transgenic mice were produced by pronuclear microinjection at the M. D. Anderson Cancer Center Genetically Engineered Mouse Facility. LSL-KRas^{G12D} and p53$^{f/f}$ conditional deletion mice were obtained from the Mouse Models for Human Cancer Consortium Repository (Rockville, MD).[8,9] All animal experiments were approved by the Institutional Animal Care and Use Committees of the University of Texas M. D. Anderson Cancer Center.

Ras Activity Assay

Total Ras activity was measured in cells lysed with beads coated with Raf1-RBD (Upstate Biotechnology, Lake Placid, NY).

Western Blot Analysis

To evaluate the levels of Ras, actin, RPS6, total Erk, p-Erk, p-AKT, p53, and DEC1 expression, cell or tissue lysates were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and immunodetection was performed as described in Supplementary Materials and Methods.

Activation of K-Ras Expression in Primary Pancreatic Acinar Cells With Cre-Bearing Adenovirus In Vitro

Primary pancreatic acinar cells were prepared and incubated with adenoviruses at a titer that was previously shown to be nearly 100% efficient and incubated at 37°C overnight.

Histology and Immunohistochemistry

Mice pancreata were fixed in 10% formalin and embedded in paraffin. H&E staining was performed in the M. D. Anderson Cancer Center Histology Core. Immunohistochemistry was performed using the RTU Vectastain Elite ABC Universal Kit (Vector Laboratories, Burlingame, CA).

β-Galactosidase Activity Assays

Frozen sections were fixed in 2% formaldehyde/0.2% glutaraldehyde in phosphate-buffered saline for 5 minutes and incubated with X-gal–containing reaction mixture provided by the manufacturer (Millipore, Billerica, MA).

Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate Nick-End Labeling Assay

To evaluate apoptotic cells, we used the In Situ Cell Death Detection Kit according to the manufacturer's suggestions (Roche, Indianapolis, IN).

Periodic Acid–Schiff and Sirius Red Staining

The mucin and collagen expression was stained with periodic acid–Schiff reagents and Sirius Red staining (Sigma Chemical Co, St. Louis, MO).

Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA preparation and quantitative reverse-transcription polymerase chain reaction (RT-PCR) were performed as we previously described.[10]

Results

Targeted Expression of Endogenous Levels of K-Ras^{G12D} in Pancreatic Acinar Cells Does Not Lead to PDAC Unless Accompanied by Loss of p53

To evaluate the effect of Ras activity specifically in pancreatic acinar cells, we utilized mice in which a tamoxifen-inducible Cre recombinase is expressed in differentiated acinar cells by a full-length mouse pancreatic elastase I gene promoter (Ela-CreERT).[11] These mice exhibit tamoxifen-independent Cre activity after birth specifically in acinar cells with high levels of elastase expression, as has been fully described previously.[11] Therefore, in the absence of tamoxifen, Cre activity was restricted to well-differentiated acinar cells. These mice were crossed with LSL-KRas^{G12D} mice, which express mutant K-Ras^{G12D} gene from the endogenous K-Ras promoter after Cre recombination.[8] Double-transgenic mice (LSL-KRas/Ela-CreERT) in the absence of tamoxifen developed typical mPanIN-1 lesions surrounded by limited focal fibrosis beginning within 2–3 months (Figure 1*A*). The mPanINs occurred infrequently in younger animals (~1 or 2 per section), but their number increased moderately over time (Figure 1*B*). These lesions were similar to those described when developmental promoters were used to activate expression

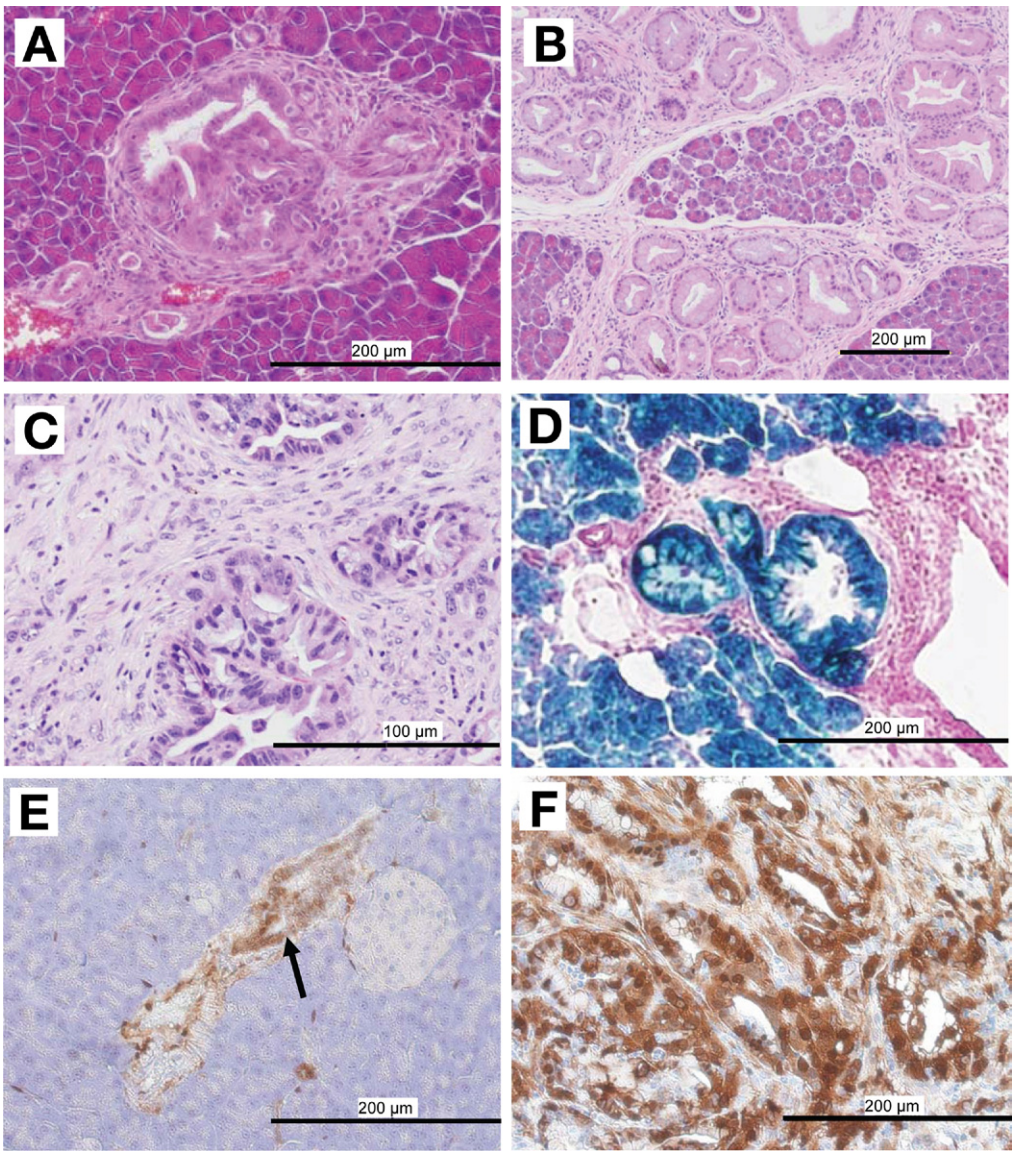

Figure 1. Targeted expression of K-Ras^{G12D} to pancreatic acinar cells at endogenous levels induced nonprogressing mPanIN-1 lesions unless p53 was deleted. (*A*) mPanIN lesions developed in a 2-month-old LSL-KRas/Ela-CreERT mouse. (*B*) The number of mPanINs increased as the mice aged but did not progress (15 months old). (*C*) Mice (LSL-KRas/p53$^{f/f}$/Ela-CreERT) that possessed endogenous expression of mutant K-Ras and p53 deletion readily developed PDAC (5 months old). (*D*) For lineage tracing, LSL-KRas/Ela-CreERT mice were further crossed with Rosa26 reporter mice such that cells of acinar lineage stained for β-galactosidase activity (*blue*). All mPanINs cells stained blue. (*E*) mPanIN cells (*arrow*) had elevated levels of p-Erk compared with surrounding acinar cells in a 4-month-old LSL-KRas/Ela-CreERT mouse detected using an anti–p-Erk antibody. (*F*) Cancer cells in PDAC developed from LSL-KRas/p53$^{f/f}$/Ela-CreERT mice (5 months old) also showed strong p-Erk staining.

of endogenous levels of mutant K-Ras.[2] However, unlike what was observed using developmental promoters, these mPanIN-1 lesions did not progress to higher-grade mPanINs or PDAC within 1.5 years of age (15/15). Yet, when the LSL-KRas/Ela-CreERT mice were further crossed with conditional p53 deletion mice (LSL-KRas/p53$^{f/f}$/Ela-CreERT), most of these mice developed PDAC within ~6 months (17/30) (Figure 1*C*). Therefore, acinar cells are readily transformed by endogenous expression of mutant K-Ras but only after a second genetic hit.

To determine the cellular origin of the mPanIN lesions in this model, these mice were further crossed with Rosa26R reporter mice (R26/LSL-KRas/Ela-CreERT). In these triple-transgenic mice, mPanIN cells showed β-galactosidase activity, indicating the acinar lineage of mPanIN lesions (Figure 1*D*). We also observed elevated levels of p-Erk in mPanIN (Figure 1*E*) and PDAC cells (Figure 1*F*) compared with acinar cells, which expressed endogenous levels of mutant K-Ras (Figure 1*E*). These data suggested that Ras activity was spontaneously upregulated during PDAC development.

Elevated Ras Activity in PDAC Cells Can Be Mimicked by Transgenic Expression of Mutant K-Ras

We next examined the level of Ras activity in mouse models and human PDAC cell lines. Ras activity was moderately elevated in most of the acinar cells in pancreatic tissues expressing a single copy of mutant K-Ras (Figure 2*A*), similar to what was previously reported using a developmental promoter.[2] In comparison, Ras activity was much more highly elevated in PDAC cell lines derived from tumors formed in LSL-KRas/p53$^{f/f}$/Ela-CreERT mice (Figure 2*A*). Likewise, signaling molecules downstream from Ras, including p-Erk, were also more highly elevated in tumor cells

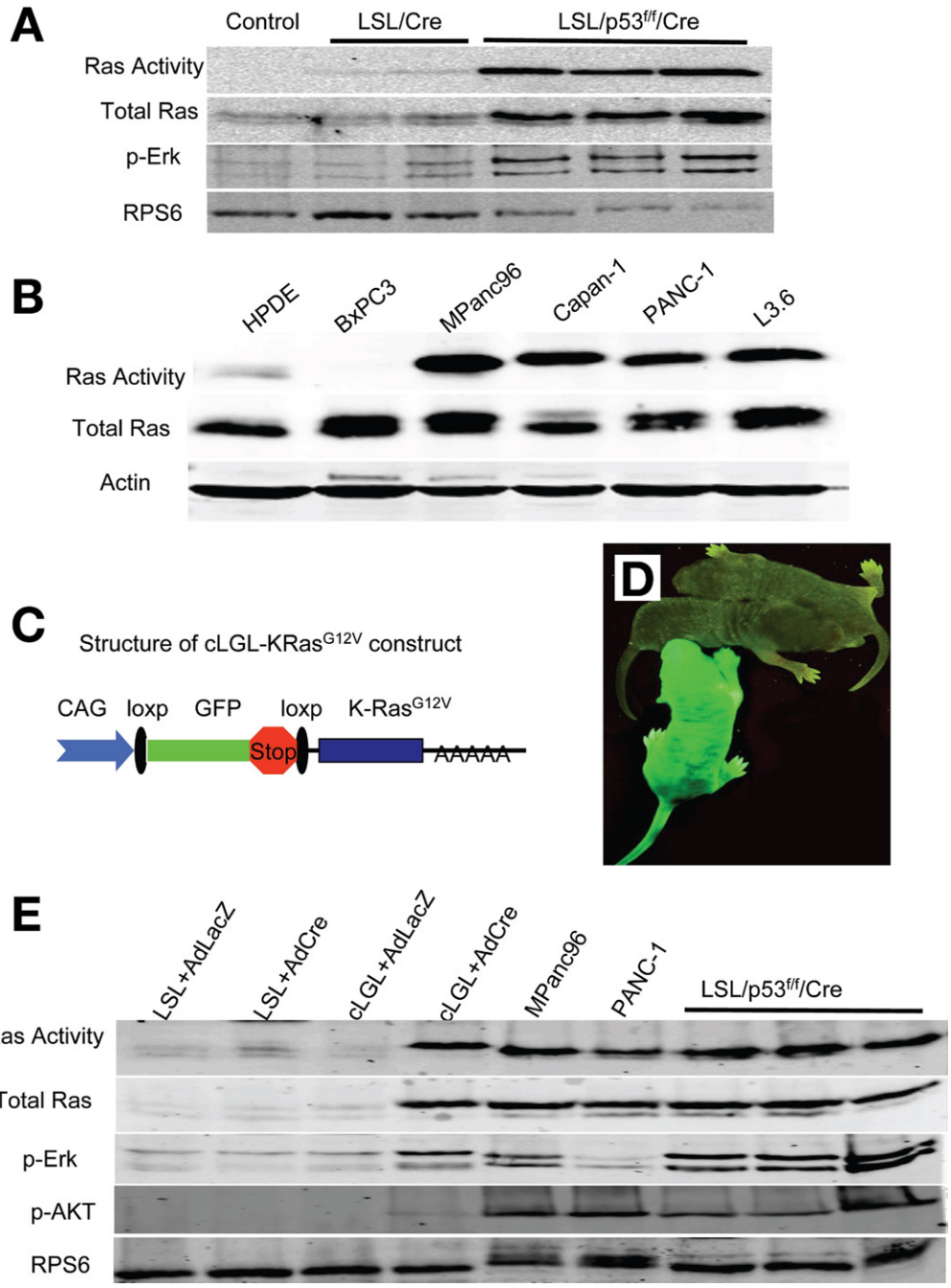

Figure 2. Transgenic expression of mutant K-Ras mimicked the elevated Ras activity levels found in pancreatic cancer cells. (*A*) Total Ras activity and p-Erk increased in PDAC cell lines isolated from LSL-KRas/p53$^{f/f}$/Ela-CreERT (LSL/p53$^{f/f}$/Cre) mice compared with untransformed pancreas tissue lysate from LSL-KRas/Ela-CreERT (LSL/Cre, 5 months old) and wild-type control (Control) mice. Deletion of p53 alone had no effect on Ras activity (data not shown). Ribosomal protein S6 (RPS6) was detected as a protein loading control. (*B*) Ras activity in human PDAC cells with K-Ras mutations (MPanc96, Capan-1, PANC-1, and L3.6) was significantly higher than that in immortalized human pancreatic duct cells (HPDE) and PDAC cells without K-Ras mutation (BxPC3). (*C*) Structure of the cLGL-KRas^{G12V} ectopic expression transgene. K-Ras^{G12V} was engineered following a human cytomegalovirus and chicken ß-actin chimeric promoter (CAG) and blocked by the proximal insertion of a loxp-GFP-stop-loxp cassette. (*D*) GFP was expressed in the whole body of cLGL-KRas^{G12V} mouse (*green*) but not in littermate controls (*gray*). (*E*) Acini isolated from LSL-KRas^{G12D} and cLGL-KRas^{G12V} mice were infected either with a control adenovirus (AdLacZ) or with an adenovirus expressing Cre (AdCre). Ras activity was analyzed by Raf pull-down and compared with those in human PDAC cell lines or mouse PDAC cell lines isolated from LSL-KRas/p53$^{f/f}$/Ela-CreERT (LSL/p53$^{f/f}$/Cre) mice. Total Ras, p-Erk, p-AKT, and RPS6 were measured in Western blots from sample aliquots before the pull-down assay.

than in nontransformed cells with endogenous levels of mutant K-Ras. Human PDAC cell lines with K-Ras mutations also exhibited very high Ras activity (Figure 2*B*). These data further support the hypothesis that Ras activity is spontaneously up-regulated during the development of PDAC, as has previously been suggested.[2]

To directly study the effects of higher Ras activity, we developed a new transgenic model in which K-Ras^{G12V} was engineered following a human cytomegalovirus and chicken ß-actin chimeric promoter (CAG)[12] and blocked by the proximal insertion of a loxp-green fluorescent protein (GFP)-stop-loxp cassette (cLGL-KRas^{G12V}; Figure 2*C*). Transgenic mice carrying this construct expressed GFP in the whole body (Figure 2*D*) before Cre recombination. The levels of Ras activity were compared between freshly prepared acinar cells isolated from LSL-KRas^{G12D} and cLGL-KRas^{G12V} mice in vitro by infecting the cells with a Cre-bearing adenovirus (AdCre). Under these conditions, the Ras activity measured in the acinar cells from the cLGL-KRas^{G12V} mice after Cre-mediated recombination was higher than that from LSL-KRas^{G12D} mice but was comparable to that observed in

human and mouse PDAC cell lines (Figure 2*E*). Likewise, levels of p-Erk and p-AKT, indicators of Ras pathway activity, were also comparable between the cLGL-KRas^{G12V} cell and human PDAC cells but higher than those observed in the LSL-KRas^{G12D} cells (Figure 2*E*). Thus, transgenic expression of mutant K-Ras achieved levels of Ras activity and downstream signaling that were similar to those observed in mouse and human PDAC cells.

Higher Levels of Acinar Cell Ras Activity Induce Inflammation and Fibrosis That Mimic the Histologic Features of Human CP

To study the effects of a higher level of Ras activity in the pancreas, cLGL-KRas^{G12V} mice were crossed with the Ela-CreERT mice, and pups were allowed to develop in the absence of tamoxifen treatments. Five days after birth, the pancreata from double-transgenic mice showed few obvious differences in size, shape, or color from littermate controls. Histologically these pancreata showed some signs of acinar-to-ductal metaplasia but were otherwise normal (Figure 3*A*). From 6 days, the pancreata from double-transgenic mice became firmer and bigger than those of the littermate controls. In adult mice, the pancreata became smaller than those of controls (Supplementary Figure 1). Histologically, changes in the pancreas in these mice as they aged involved a progression from normal pancreas to extensive fibrosis resembling CP (Figure 3*A*–*C*). CP was characterized by loss of acinar cells, acinar-to-ductal metaplasia, leukocyte infiltration, and replacement by stroma with collagen deposition (Supplementary Figure 1*C*). Cells observed within the stroma displayed pronounced smooth muscle actin staining (Figure 3*D*), which suggested that they were activated stellate cells.[13]

The loss of acinar cells that occurred in this model was associated with the expression of senescence-associated β-galactosidase (Figure 4*A*), with little evidence of apoptosis (Figure 4*B*). DEC1, another marker of senescence,[14] was dramatically increased early in this model (Figure 4*C*). Tumor suppressor genes p53, p15, and p16, which are known to be involved in oncogene-induced senescence, were also elevated (Figure 4*C* and *D*). Senescent cells induced by oncogenic Ras in vitro have been reported to develop a senescence-associated secretory phenotype, which results in the secretion of inflammatory cytokines.[15] We found in this in vivo model that high levels of Ras activity induced increased expression of interleukin-1β, interleukin-6, Gro-α, and granulocyte-macrophage colony–stimulating factor (Figure 4*F*).

Elevated Levels of Ras Activity in Acinar Cells Result in Rapid Development of mPanIN, CPC, and PDAC

In the background of CP, we observed the formation of multifocal acinar-to-ductal metaplasia in the pan-

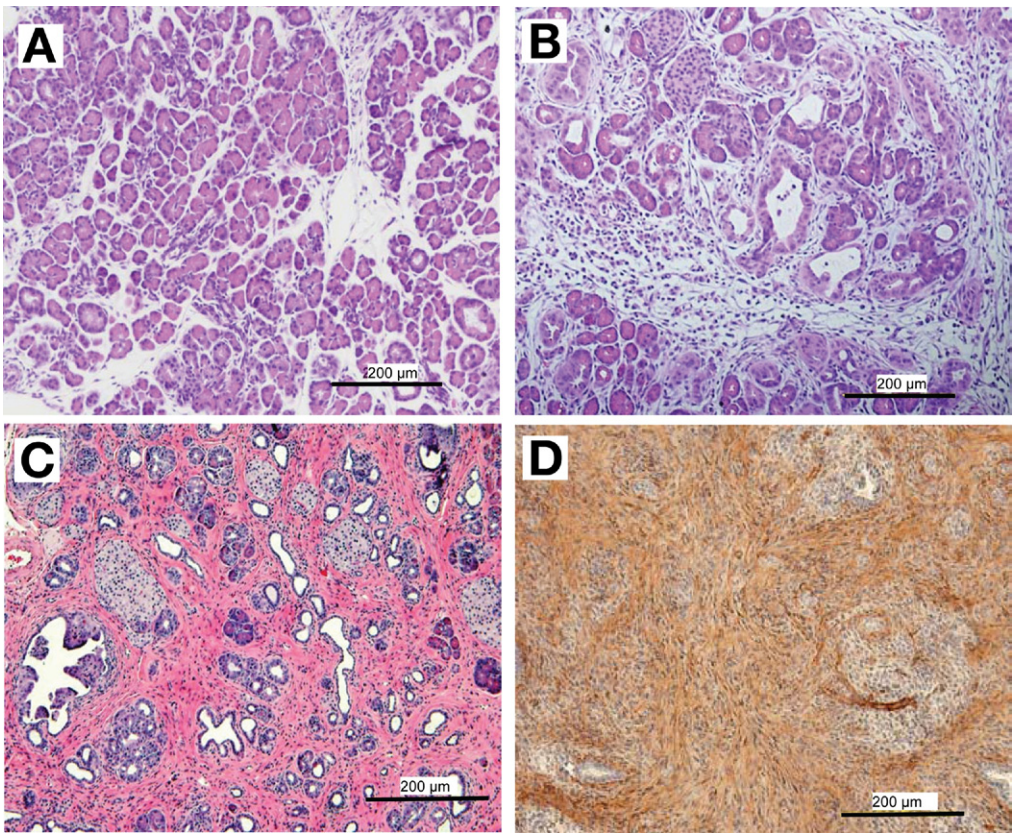

Figure 3. High levels of Ras activity in pancreatic acinar cells led to the development of CP. (*A*) At 5 days after birth, pancreata from cLGL-KRas/Ela-CreERT mice showed normal histology except for occasional sites of acinar-to-ductal metaplasia. (*B*) At 9 days after birth, acinar cells were depleted from many areas with the development of fibrotic stroma. (*C*) In adult mice (2 months), the fibrosis expanded with abundant intercellular collagen deposition. (*D*) The stromal compartment of the pancreas (2 weeks old) showed abundant smooth muscle actin expression.

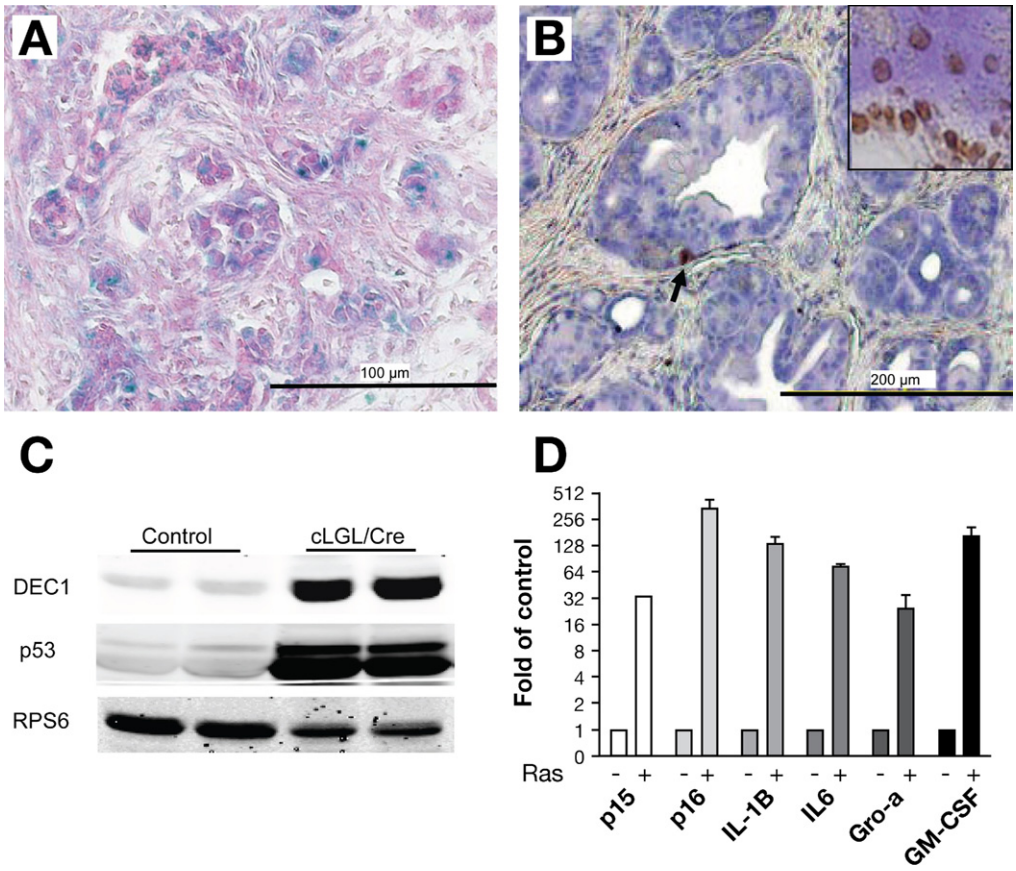

Figure 4. Elevated Ras activity in pancreatic acinar cells caused acinar cell senescence and stellate cell activation. (*A*) In adult cLGL-KRas/Ela-CreERT mice (2 months old), many acinar cells stained positive for senescence-associated β-galactosidase. (*B*) Few pancreatic acinar cells showed apoptosis (*arrow*) as indicated by terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay (2 months old). Deoxyribonuclease-treated tissue served as a positive control (*inset*). (*C*) DEC1 and p53 were up-regulated in cLGL-KRas/Ela-CreERT (cLGL/Cre) mice (2 months old) as measured by Western blot. (*D*) Quantitative reverse-transcription polymerase chain reaction showed that p15, p16, interleukin-1β, interleukin-6, Gro-α, and granulocyte-macrophage colony–stimulating factor were up-regulated (shown in $\log_2$ scale) in cLGL-KRas/Ela-CreERT mice (1 month old) as compared with wild-type littermate controls.

creas from all (100%) cLGL-KRas^{G12V}/Ela-CreERT mice (Figure 5*A*). In younger mice, we observed primarily mPanIN-1 (Figure 5*B*) and occasionally mPanIN-2 lesions (Figure 5*C*). As the animals aged, there was an increase in the level of dysplasia and in the frequency of mPanIN-3 lesions (Figure 5*D*). Three out of 20 mice developed noninvasive CPC, which were composed of large cystic lesions (>1.0 mm) lined with highly dysplastic ductal epithelial cells forming finger-like papillae (Figure 5*E*). The frequencies of mPanIN lesions of different histologic grades developed by different age groups are shown in Figure 5*F*. Acinar-to-ductal metaplasia and mPanINs in these mice exhibited typical characteristics similar to what were observed in other models (Supplementary Figure 2*A*–*F*).[2]

At 4 months of age, one mouse developed PDAC (Figure 6*A*) with metastasis to the liver (Supplementary Figure 2*G*) and lungs (data not shown). By 6 months of age, 29% of mice (7/24) developed PDAC. At 9 months, the incidence for PDAC increased to 50% (10/20). Metastatic PDAC cells were also identified in peripancreatic lymph nodes (Figure 6*B*). Some mice showed extensive tumor invasion into the diaphragm (Figure 6*C*). All tumors were adenocarcinomas composed of neoplastic glands, similar to those seen in human PDAC. No staining of the acinar cell marker, amylase, was detected by immunohistochemistry (Supplementary Figure 2*H*). The tumors were moderately to poorly differentiated, with 10%–40% desmoplastic tumor stroma. Negative staining for GFP supported that the PDAC cells originated from pancreatic acinar cells (Figure 6*D*).

To further evaluate the levels of Ras activity in PDAC cells developed from different models, we measured Ras activity in cancer cell lines developed from LSL-KRas/p53$^{f/f}$/Ela-CreERT and cLGL-KRas/Ela-CreERT mice as well as human PDAC cell lines (Figure 6*E*). We observed comparable levels of Ras activity in each of these cancer cell lines, suggesting that Ras activity progressed to peak levels in PDAC cells even when the cancers were initiated with endogenous levels of mutant K-Ras expression. All 4 independent cancer cells from cLGL-KRas/Ela-CreERT mice lost expression of p15 and p16 but retained p53 (Figure 6*F*). These p53 were verified as wild-type by DNA sequencing. Conversely, cancer cells from LSL-KRas/p53$^{f/f}$/Ela-CreERT mice lacked p53 expression as expected but retained intact p15 and p16 (Figure 6*F*).

Discussion

In this study, we investigated the importance of Ras activity levels in the development of PDAC. We showed that the level of Ras activity in PDAC cells, whether mouse or human, is highly elevated. We confirmed that adult pancreatic acinar cells are largely re-

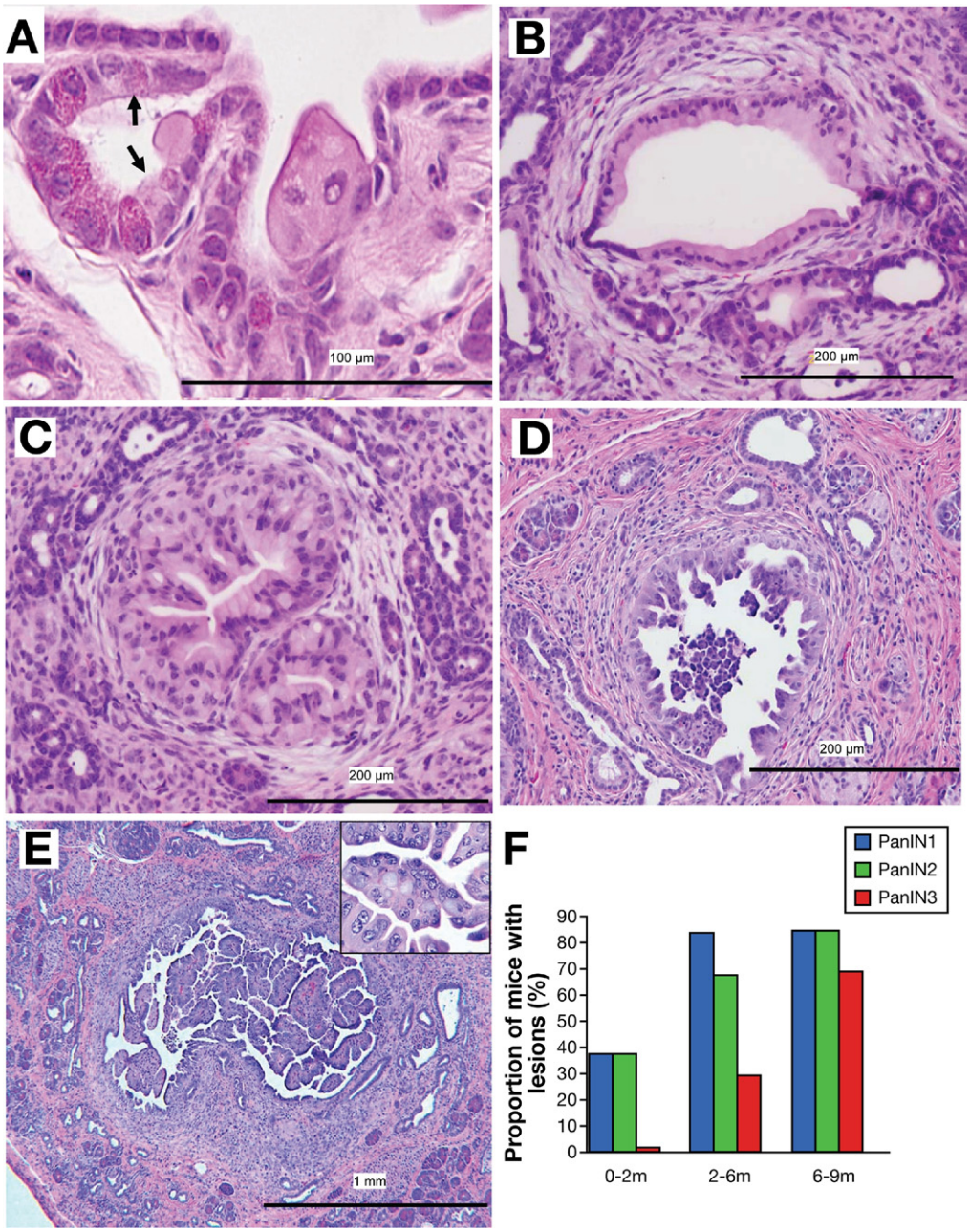

Figure 5. High levels of Ras activity caused acinar-to-ductal metaplasia and led to the formation of mPanINs and CPC. (*A*) In cLGL-KRas/Ela-CreERT mice (2 months old), acinar-to-ductal metaplasia (*arrows*) was observed. (*B–E*) Transgenic expression of mutant K-Ras induced typical mPanINs that progressed over time, including mPanIN-1 (*B*, 2 months), mPanIN-2 (*C*, 4 months), and mPanIN-3 (*D*, 6 months) lesions and CPC (*E*, 7 months). (*F*) The grade of the mPanIN lesions present in the pancreas of transgenic animals progressed with age. Each group consisted of ~20 animals.

fractory to endogenous levels of mutant K-Ras[4,16,17] and showed for the first time that this resistance can be readily overcome by deletion of p53. Furthermore, we observed that levels of Ras activity that mimicked the levels found in PDAC cells had profound effects on acinar cells that have not been reported previously, including the spontaneous development of CP, CPC, and PDAC. Therefore, our data indicate that the levels of Ras activity are of great importance in the development of pancreatic diseases.

It was previously reported that levels of Ras activity were significantly elevated in bulk tumor tissues from mice expressing endogenous levels of K-Ras, although it was unclear if the elevated activity reflected increased signaling in cancer cells or an alteration in the balance of cell types in the tumors.[7] In the current study, we identified the cancer cells themselves as the source of this high Ras activity. We also observed that both mouse and human PDAC cells possessed greatly elevated Ras activity compared with untransformed cells expressing endogenous levels of mutant K-Ras. When these high levels of Ras activity were directly mimicked by transgenic expression of mutant K-Ras in pancreatic acinar cells, we observed the development of senescence, diffuse CP, acinar-to-ductal metaplasia, multifocal mPanINs, and a high incidence of PDAC at a relatively early age. These data support the concept that a threshold of Ras activity is important for the induction of cellular transformation

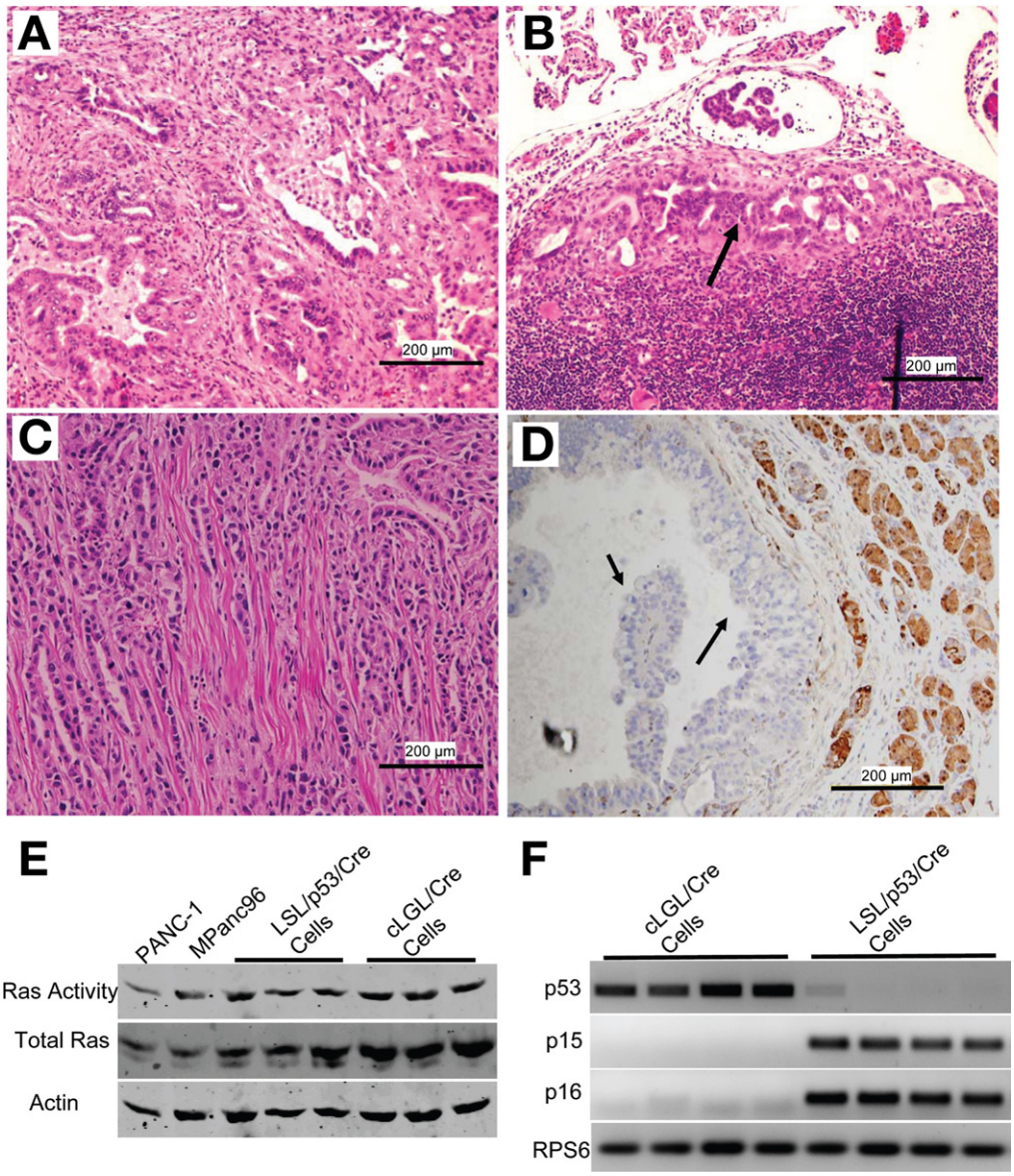

Figure 6. Invasive and metastatic PDAC developed spontaneously from pancreatic acinar cells with levels of Ras activity that mimicked those of human PDAC. (*A*) PDAC developed in cLGL-KRas/Ela-CreERT mice (4 months). (*B*) Metastatic cancer cells (*arrow*) were observed in the lymph nodes. (*C*) Some PDAC invaded the diaphragm. (*D*) GFP expression was lost in cancer cells (*arrows*), suggesting they were of pancreatic acinar origin. (*E*) Ras activity was measured in 3 independent cancer cell lines developed from LSL-KRas/p53$^{f/f}$/Ela-CreERT (LSL/p53/Cre Cells) mice and cLGL-KRas/Ela-CreERT (cLGL/Cre Cells) and compared with those found in human PDAC cells. (*F*) Reverse-transcription polymerase chain reaction measurement showed that cancer cells from cLGL-KRas/Ela-CreERT (cLGL/Cre Cells) mice lost expression of p15 and p16 but retained p53. In contrast, cancer cells from LSL-KRas/p53$^{f/f}$/Ela-CreERT (LSL/p53/Cre Cells) mice lost p53 expression but retained intact p15 and p16.

and that this level can be achieved spontaneously in models with endogenous levels of mutant K-Ras expression[6] or directly by expression of higher levels of mutant K-Ras.

The mechanisms responsible for the stochastic events resulting in the elevation of Ras activity in cells expressing mutant K-Ras from the endogenous promoter are currently unknown. It may be partly explained by the common phenomenon of amplification of the mutant K-Ras allele.[7] However, other alterations in signaling pathways may also contribute to the increased Ras activity independently or in addition to K-Ras mutation. For example, it has also been observed that combining low levels of expression of mutant K-Ras with other stimuli known to increase Ras activity such as transforming growth factor (TGF)-α[18] or cholecystokinin[4] leads to much more profound phenotypes. This supports the concept that up-regulated Ras pathway activity by extrinsic stimuli may also play an important role in transformation.

The current study confirmed previous observations that low endogenous levels of Ras activity are insufficient to transform fully differentiated pancreatic acinar cells.[4,16,19] However, it also shows for the first time that combination of p53 deletion with endogenous levels of mutant K-Ras in acinar cells readily leads to PDAC. Furthermore, we observed that adult acinar cells were spontaneously transformed by elevated levels of mutant K-Ras expression that resulted in levels of Ras activity similar to those observed in PDAC cells. Taken together, these data support that pancreatic acinar cells can be a cell origin of PDAC, providing elevated Ras activity and loss of tumor suppressor genes occurs.

In 2 previous studies in which endogenous mutant K-Ras expression was initiated by elastase[4] or pdx1 promoters,[20] CP induced by secretagogue administration was found to accelerate PDAC formation. This study differs from previous ones in that higher levels of Ras activity drove both CP and spontaneous PDAC in the absence of additional treatments. Thus, Ras activity above a threshold is sufficient for both inflammatory and transforming responses. Of note, the treatments commonly utilized to induce pancreatitis also activate Ras.[21] Therefore, one possible explanation for the previous observations is that the treatments used to generate inflammation themselves elevated the level of Ras activity above a threshold leading to the observed effects.

These data support a model in which there are 2 significant barriers to PDAC development, as have been previously proposed in mammary cancer.[6,22] One barrier is the elevation of levels of Ras activity beyond a critical threshold. This barrier can be overcome either by increased extrinsic Ras activators, such as coexpression of TGF-α[23] or administration of cholecystokinin,[4,20] or, as in the current study, by elevated expression of mutant K-Ras. Yet, even with high levels of Ras activity, there remains a latency of several months before tumors form. Furthermore, elevated Ras activity primarily drives cellular senescence rather that tumorigenesis. Thus, another barrier, the loss of tumor suppressors, is required for evasion of oncogene-induced senescence and full transformation. As shown here and in other studies, deletion or mutation of p53,[24] p16,[7] or p19[25] can dramatically accelerate tumor formation. These 2 barriers are not independent. Loss of tumor suppressors is permissive for cells with spontaneously up-regulated Ras activity to evade oncogene-induced senescence. It also appears that the spontaneous loss of tumor suppressors is accelerated by high levels of Ras activity, likely due to the associated increase in genetic instability.[26]

Another major finding in this study was that Ras activity at levels similar to those observed in PDAC cells drove the development of an inflammatory phenotype similar to the histologic features of human CP. This observation raises an issue concerning the commonly observed presence of K-Ras mutations in CP. It has been well recognized that K-Ras mutations are present in nearly all PDAC.[27,28] However, it is less well appreciated that K-Ras mutations are also found in nearly 30%–40% of patients with CP.[29] The biological significance of K-Ras mutations in CP has not been understood. One popular hypothesis is that prolonged inflammatory conditions of CP increase the probability of gene mutations, as has been reported.[30] However, K-Ras mutations have also been observed in hyperplastic ducts within normal pancreas.[31] Therefore, it is likely that K-Ras mutations can occur before the development of CP. In the current study, we observed that elevation of Ras activity in pancreatic acinar cells led directly to CP. Thus, our study provides evidence of a potential functional relationship between K-Ras mutations and CP in which mutant K-Ras is a cause rather than a secondary effect of CP. The present study also suggests that the activity level, rather than the presence of mutations, is the critical parameter. It is intriguing to speculate that the inflammatory response observed in this study may have been associated with the induction of senescent cells by high levels of Ras activity. Senescent cells are known to secrete myriad factors associated with inflammation.[15] This animal model should be useful for future studies to elucidate the senescence-associated secretory phenotype and the development of fibrosis in vivo. It is noted that not all pancreases of patients with CP possess K-Ras mutations. However, it is known that the Ras signaling pathway is activated by several stimuli known to cause CP in mouse models, including cholecystokinin treatments,[21] expression of cyclooxygenase-2,[32] and expression of TGF-α.[33] Thus, we speculate that increases of Ras activity in pancreatic acinar cells brought about by various treatments may mediate the development of fibrosis and inflammation even in the absence of K-Ras mutations. Whether these concepts apply to the human disease will require further investigation.

We also observed that acinar cells gave rise to CPC in the mouse model with higher levels of Ras activity. This

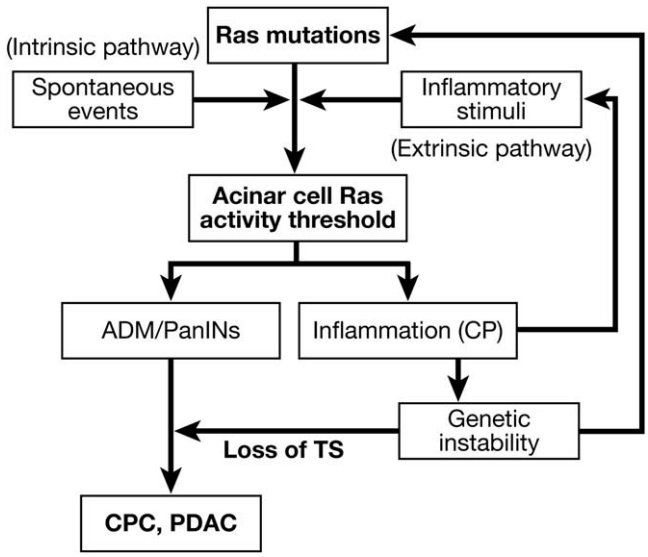

Figure 7. Ras activity levels control the development of pancreatic diseases. For the initiation of pancreatic diseases, Ras activity levels must be elevated beyond a threshold. This can be accomplished either by high levels of extrinsic Ras activators (extrinsic pathway) or intrinsic alterations of Ras activity including activating mutations of K-Ras (intrinsic pathway) or by a combination of these 2 pathways. Once reached, levels of Ras activity above a threshold cause acinar cells to undergo acinar-to-ductal metaplasia and to form PanINs. High levels of Ras activity also lead to the generation of inflammation resembling CP. Inflammation-induced genetic instability likely increases the probability of genetic alterations including mutations in K-Ras and loss of tumor suppressors (TS), which are required for the development of PDAC.

is the first evidence that acinar cells can give rise to this pathological condition. In mouse models, cystic papillary neoplasms are defined as large (>1 mm) cystic structures composed of usually papillary, noninvasive epithelial proliferations with varying degrees of cellular atypia. These lesions resemble human intraductal papillary mucinous neoplasms,[4,34] which are considered an important precursor lesion to PDAC.[35] In a recent study in transgenic mice, it was observed that a combination of expression of mutant K-Ras with overexpression of TGF-α was able to lead to the development of structures resembling intraductal papillary mucinous neoplasm.[21] In that study, it was suggested that the CPCs did not originate from pancreatic acinar cells based on the observation that the cells in the CPCs did not express acinar-specific genes. However, in the current study, we observed that acinar cells rapidly lost differentiated gene expression upon metaplasia such that it is difficult to know the cell of origin without lineage tracing.

In summary, elevated Ras activity plays a crucial role in the development of pancreatic cancer and provides a possible direct linkage between CP, CPC, and PDAC. Based on our observations and taking into account observations from other models, we propose a scheme to explain the role of Ras activity in progression of pancreatic disease (Figure 7). According to this mechanistic view, 2 key obstacles to the formation of PDAC are achievement of a high level of Ras activity and the loss of tumor suppressors. High levels of Ras activity also generate an inflammatory response resembling CP that likely accelerates genetic changes. The animal model described in this study will provide a powerful tool for further investigations of the mechanisms underlying CP, pancreatic cancer, and the transition between these diseases.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.05.052.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Genetically Engineered Mice

To generate cLGL-KRas^{G12V}, a fragment containing loxP-GFP-stop-loxP followed by K-Ras^{G12V} was cloned into pCAGGS vector (gift from Dr Miyazaki, Kumamoto University Medical School, Kumamoto, Japan), which contains a cytomegalovirus (CMV) and chicken β-actin chimeric promoter.[1] For pronuclear injection, cLGL-KRas^{G12V} was digested with *Spe*I and *Hin*dIII to remove vector sequences and the cLGL-KRas^{G12V} DNA fragments were purified. LSL-KRas^{G12D} mice (Donated by Dr Tyler Jacks, Massachusetts Institute of Technology, Cambridge, MA) and p53$^{f/f}$ conditional deletion mice (donated by Dr Anton Berns, Netherlands Cancer Institute, Amsterdam, The Netherlands) were obtained from the Mouse Models for Human Cancer Consortium Repository (Rockville, MD).[2,3]

Ras Activity Assay

Cells were lysed on ice in 25 mmol/L HEPES (pH 7.5) containing 1% Igepal CA-630 (Sigma, St. Louis, MO), 150 mmol/L NaCl, 10 mmol/L $MgCl_2$, 1 mmol/L EDTA, 10% glycerol, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 25 mmol/L sodium fluoride, and 1 mmol/L sodium orthovanadate and cleared by centrifugation at 15,340*g* for 5 minutes at 4°C. Aliquots of lysates were set aside to allow quantification of total Ras or protein loading controls by immunoblotting. Equal amounts of lysate protein were incubated for 45 minutes at 4°C, with beads coated with Raf1-RBD (Upstate Biotechnology, Inc, Lake Placid, NY). Beads were then washed 3 times with ice-cold lysis buffer, and bound protein was eluted for 15 minutes with 2× Laemmli sample buffer that had been preheated to 95°C and analyzed by immunoblotting for Ras following Western blot analysis protocol.

Western Blot Analysis

To evaluate the levels of Ras, actin, RPS6, total Erk, p-Erk, p-AKT, p53, and DEC1 expression, cell or tissue lysates were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and immunodetection was performed with the Odyssey fluorescent imager (Li-Cor Biosciences, Lincoln, NE).

Cell or tissue lysates were prepared, separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and transferred to nitrocellulose. Membranes were blocked for 1 hour at room temperature with 3% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST), washed, and incubated overnight at 4°C with one of the following primary antibodies: anti–K-Ras (1:10,000; Upstate Biotechnology, Inc, Lake Placid, NY), anti-actin (1:1000; Sigma Chemical Co, St Louis, MO), anti-RPS6 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), p-Erk and p-AKT antibodies (1:1000; Cell Signaling Technology, Danvers, MA), and anti-Dec1 (0.25 μg/mL; Aviva Antibody Corp, San Diego, CA). Immunodetection was performed with the corresponding Alexa Fluor 680–conjugated secondary antibodies. Fluorescence was detected with the Odyssey fluorescent imager (Li-Cor Biosciences), and images were converted to gray scale.

Activation of K-Ras Expression in Primary Pancreatic Acinar Cells With Cre-Bearing Adenovirus In Vitro

A recombinant adenovirus encoding the Cre recombinase (AdCre) was produced according to the method of He et al.[4] Briefly, a DNA fragment encoding the full-length Cre recombinase was ligated behind the CMV promoter of pAd-Track-CMV shuttle vector, producing pAd-Track-CMV-Cre plasmid. Under the control of a distinct CMV promoter, this plasmid also expresses GFP. The construct was linearized with *Pme*I and cotransfected together with the adenoviral backbone vector, pAd-Easy-1, into *Escherichia coli* strain BJ5183. Recombinant cosmids were selected with kanamycin and screened by *Bam*HI and *Pac*I digestion. The adenoviral construct was then cleaved with *Pac*I and transfected in a packaging cell line (human embryonic kidney 293 cells) to produce AdCre. An adenovirus (AdLacZ) expressing bacterial β-galactosidase and GFP, each under the control of a separate CMV promoter, was a gift from Dr He (John Hopkins Oncology Center, Baltimore, MD) and utilized as a control. The recombinant virus was concentrated using a CsCl gradient. The titer of the viral stocks was estimated based on the density of GFP-expressing cells.

Primary pancreatic acinar cells were prepared by methods previously described.[5] In brief, the pancreata from cLGL-KRas^{G12V} mice were excised, and pancreatic acini were prepared by enzymatic digestion with purified collagenase, followed by mechanical shearing. Acini were then filtered through 150-μm Nitex mesh and purified by sedimentation through 4% bovine serum albumin in Dulbecco's modified Eagle medium (DMEM). The acini were suspended in DMEM containing 1% bovine serum albumin in the presence of 0.1 mg/mL soybean trypsin inhibitor. AdCre or AdLacZ at a titer of $\sim 10^7$ plaque-forming units/mg protein were added to acini in 3 mL of DMEM for 10 minutes. The acini were then diluted with DMEM to 13 mL, transferred to 100-mm dishes, and incubated at 37°C in a humidified 5% CO_2 atmosphere overnight.

Histology and Immunohistochemistry

Mice pancreata were fixed in 10% formalin and embedded in paraffin. H&E staining was performed in the M. D. Anderson Cancer Center Histology Core. For immunohistochemistry, unstained sections were deparaffinized with xylene and rehydrated in water. Immunohistochemistry was performed using RTU Vectastain Elite ABC Universal Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Sections were treated with primary antibodies diluted in 2% bovine serum albumin/0.2% Triton X-100 in phosphate-

buffered saline (PBS) overnight followed by incubation with corresponding horseradish peroxidase–labeled secondary antibody. Finally, slides were developed with 3,3-diaminobenzidine substrate and counterstained with hematoxylin, dehydrated with ethanol, fixed with xylene, and mounted. Primary antibodies anti-GFP (1:1000) and anti–smooth muscle actin (1:100) were purchased from Abcam (Cambridge, MA). Anti–proliferating cell nuclear antigen (1:100), anti–cyclooxygenase-2 (1:50), anti-amylase (1:200), and anti–Hes-1 (1:50) antibodies were purchased from Santa Cruz Biotechnology. p-Erk antibody (1:100) was from Cell Signaling Technology.

β-Galactosidase Activity Assays

For detection of β-galactosidase activity in mice bearing the Rosa26 floxed gene,[6] frozen sections were fixed in 0.2% glutaraldehyde, 50 mmol/L ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and 100 mmol/L $MgCl_2$ in PBS for 15 minutes and then washed 3 times with PBS followed by staining in X-gal solution (pH 7.4) containing 1 mg/mL X-gal, 5 mmol/L potassium ferricyanide, 2 mmol/L potassium ferrocyanide, 2 mmol/L $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 at 37°C overnight. To measure senescence-associated β-galactosidase activity,[7] frozen sections were fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS for 5 minutes and incubated with X-gal–containing reaction mixture (pH 6.0) provided by the manufacturer (Millipore, Billerica, MA).

TUNEL Assay

To evaluate apoptotic cells, we used the In Situ Cell Death Detection Kit according to the manufacturer's suggestions (Roche, Indianapolis, IN). Basically, histologic sections were treated with proteinase K in Tris-HCl (pH 7.5) at room temperature, rinsed with PBS, and incubated in 0.3% H_2O_2 in methanol for 30 minutes. After permeabilization in 0.1% Triton X-100 in sodium citrate buffer, sections were incubated with the TUNEL reaction mixture containing terminal transferase and biotin-16-deoxyuridine triphosphate. After washing, the slides were treated with horseradish peroxidase–conjugated streptavidin followed by chromagen 3,3′-diaminobenzidine tetrahydrochloride. Positive controls were prepared by adding 1 U/μL deoxyribonuclease for 10 minutes.

Periodic Acid–Schiff and Sirius Red Staining

The mucin expression was stained with periodic acid–Schiff reagents according to the manufacturer's instructions (Sigma Chemical Co). Briefly, pancreas sections were deparaffinized, hydrated to deionized water, and then immersed in periodic acid solution for 5 minutes at room temperature. After washing with distilled water, the slides were incubated in Schiff's reagent for 15 minutes followed by counterstaining with hematoxylin solution. The glycol-containing mucin stains pink to red.

The presence of collagen was revealed with Sirius Red staining (Sigma Chemical Co). In brief, slides were deparaffinized and hydrated to water and nuclei were stained with Weigert's hematoxylin for 8 minutes. Following 10 minutes of washing in running tap water, the slides were stained in picro-Sirius Red solution containing 0.1% Sirius Red in saturated aqueous solution of picric acid for 1 hour. Collagen-rich tissue is stained red on a pale yellow background.

Reverse-Transcription Polymerase Chain Reaction and Quantitative Reverse-Transcription Polymerase Chain Reaction

Standard reverse-transcription polymerase chain reaction (RT-PCR) was conducted using total RNA prepared from mouse tissues or cell lines isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was further purified by digestion for 15 minutes with deoxyribonuclease and recovery of RNA using an RNeasy kit (Qiagen, Valencia, CA). Reverse transcription was conducted for 45 minutes at 48°C from 1 μg of purified total RNA in a 25 μL volume of Access RT-PCR reaction mixture (Promega, Madison, WI) followed by 40 cycles of standard PCR (30 seconds of denaturation at 94°C, 1 minute of annealing at 60°C, and 1.5 minutes of extension at 68°C). Quantitative RT-PCR was conducted using SYBR green I to monitor the PCR products on the I-Cycler thermal cycler and IQ real-time PCR detection system (Bio-Rad, Hercules, CA). Primers were designed to span at least one intron to further eliminate the influence of genomic DNA contamination in the RNA preparation. RPS6 gene was used as an internal RNA loading control. Primers sequences were as follows: p15 (GenBank accession NM_007670; forward, CGGCGAAGGACCATTTCTG; reverse, GTTCAGGGCGTTGGGATC), p16 (GenBank accessionNM_009877; forward, GTGCGATATTTGCGTTCC; reverse, CTCTGCTCTTGGGATTGG), p53 (GenBank accession NM_011640; forward, TGAACCGCCGACCTATCCTTACC; reverse, CCCAGGGCAGGCACAAACAC), p53 full length (GenBank accession NM_011640; forward, CTTCCCAGCAGGGTGTCACGCT; reverse, GGACGGGATGCAGAGGCAGTCA), interleukin-1β (GenBank accession NM_008361; forward, GCACTACAGGCTCCGAGATG; reverse, GTCGTTGCTTGGTTCTCCTTG), interleukin-6 (GenBank accession NM_031168; forward, AGACAAAGCCAGAGTCCTTCAG; reverse, AGCCACTCCTTCTGTGACTCC), Gro-α (GenBank accession NM_008176; forward, CGAAGTCATAGCCACACTCAAG; reverse, CCAGACGGTGCCATCAGAG), granulocyte-macrophage colony–stimulating factor (GenBank accession NM_009969; forward, AGACCCGCCTGAAGATATTCG; reverse, CCGCATAGGTGGTAACTTGTG), and RPS6 (GenBank accession NM_009096; forward, GATGTCCGCCAGTATGTTGTC; reverse, TCTTAGTGCGTTGCTTCTTCAG).

To detect possible mutations of p53 gene in cLGL-KRas^{G12V}/Ela-CreERT mice, we directly sequenced the

open reading frames for p53 after RT-PCR amplification of full-length p53 from isolated PDAC cell messenger RNA.

Supplementary Results

Inflammation Developed in cLGL-KRas/Ela-CreERT Mice

From 6 days, the pancreata from cLGL-KRas/Ela-CreERT double-transgenic mice became firmer and bigger than those of the littermate controls (Supplementary Figure 1*A*). In adult mice, the pancreata became smaller than those of controls (Supplementary Figure 1*B*). Histologically, normal pancreas was replaced by stroma with collagen deposition (Supplementary Figure 1*C*).

Characteristics of mPanINs in cLGL-KRas/Ela-CreERT Mice

High mucin content was observed in mPanINs (Supplementary Figure 2*A*). Focal mucin staining was also observed in cells with acinar morphology in regions of acinar-to-ductal metaplasia, indicating that mucin expression is an early event in the transitional state of acinar cells undergoing metaplasia (Supplementary Figure 2*A*, *arrows*). As in previous models, mPanIN cells showed activated Notch signaling, which was indicated by nuclear staining of transcription factor Hes1 (Supplementary Figure 2*B*). Senescence-associated β-galactosidase was expressed in mPanINs (Supplementary Figure 2*C*), suggesting that oncogene-induced senescence may be crucial to limit mPanIN progression and cancer development. In mPanINs, most cells lost expression of the pancreatic acinar cell marker amylase (Supplementary Figure 2*D*). p-Erk was increased in mPanIN cells (Supplementary Figure 2*E*) and cancer cells (Supplementary Figure 2*F*) developed from cLGL-KRas/Ela-CreERT mice.

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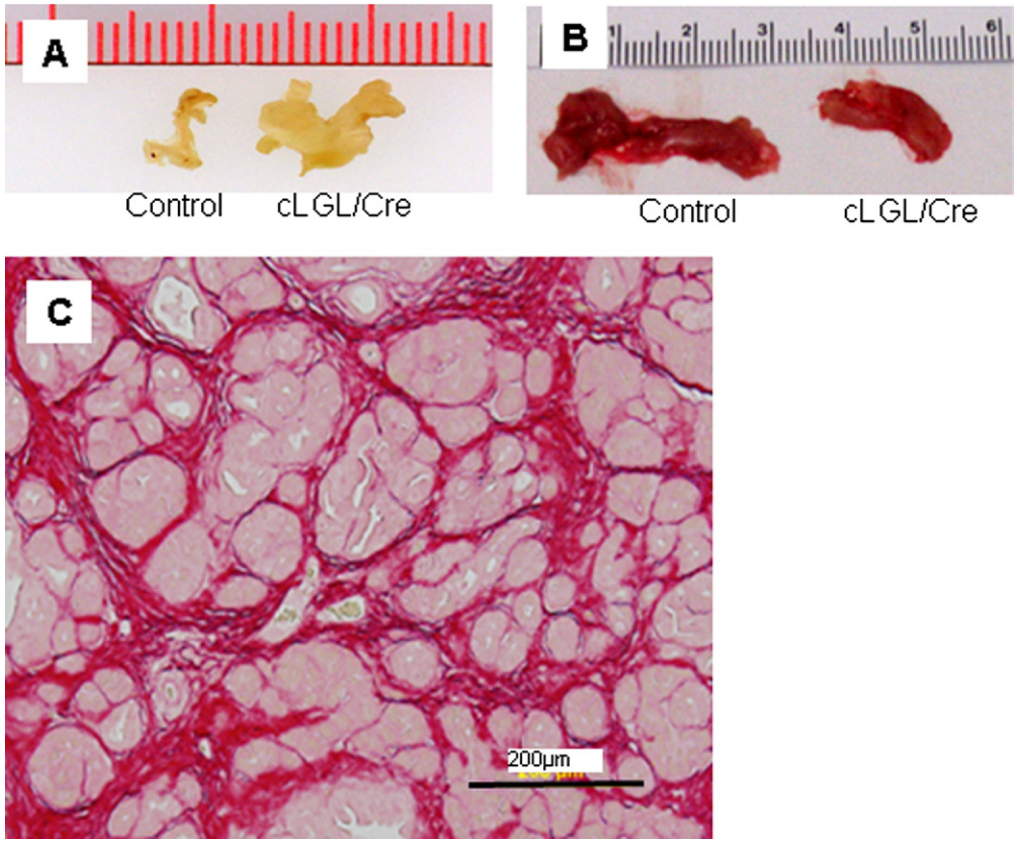
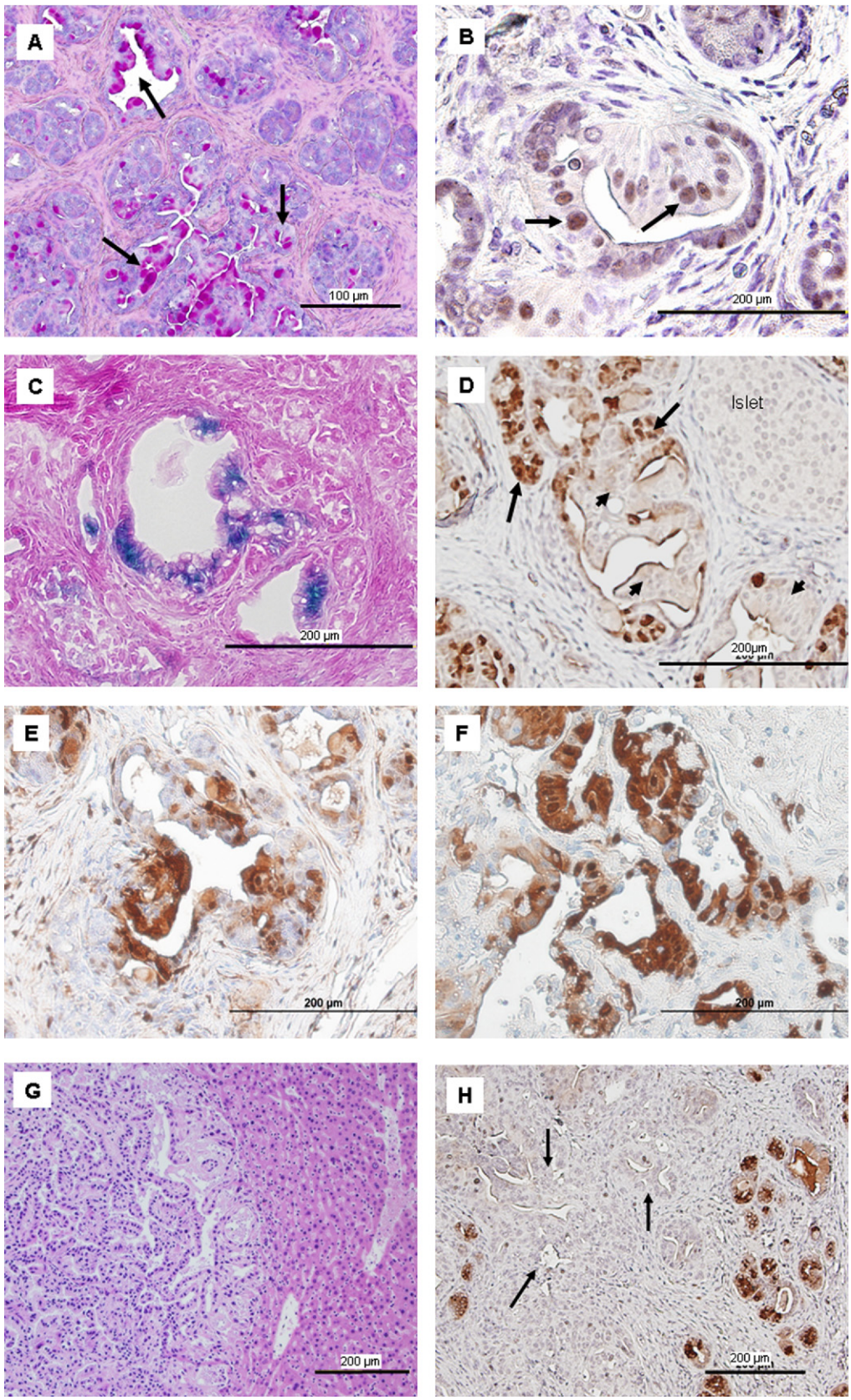


Supplementary Figure 1. High level of Ras activity in pancreatic acinar cells induced CP. (*A*) Pancreata (formalin-fixed) from cLGL-KRas/Ela-CreERT (cLGL/Cre) double-transgenic mice were bigger and firmer (*right*) than those from wild-type mice (*left*) at 2 weeks after birth. (*B*) Pancreata (without fixation) from cLGL-KRas/Ela-CreERT (cLGL/Cre) double-transgenic mice were smaller and firmer (*right*) than those from wild-type mice (*left*) in adult mice. (*C*) The abundant stroma in the pancreatic tissue of double-transgenic mice (2 months old) showed pronounced staining for collagen with Sirius Red.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Supplementary Figure 2. Acinar-to-ductal metaplasia and mPanINs in the transgenic model with high levels of mutant K-Ras exhibited typical characteristics. (*A*) In adult cLGL-KRas/Ela-CreERT double-transgenic mice (2 months old), periodic acid–Schiff (PAS) staining indicated abundant mucin (*red*) in ductal structures and acinar cells undergoing acinar-to-ductal metaplasia (*arrows*). (*B*) Hes1, a Notch-signaling target gene normally present during pancreatic development, was up-regulated and localized to the nucleus (*brown*) in mPanINs (*arrows*). (*C*) Senescence, indicated by senescence-associated β-galactosidase expression, was present in mPanINs (*blue*). (*D*) Amylase expression, a marker of differentiated acinar cells, was analyzed using an anti-amylase antibody in sections taken from a cLGL-KRas/Ela-CreERT mouse pancreas. This acinar cell marker was lost in cells within mPanINs (*arrowheads*) but not in adjacent acinar cells (*arrows*). (*E*) p-Erk was stained by immunohistochemistry in mPanIn cells from a cLGL-KRas/Ela-CreERT mouse. (*F*) PDAC developed from a cLGL-KRas/Ela-CreERT mouse showed strong p-Erk staining. (*G*) PDAC developed in cLGL-KRas/Ela-CreERT mice (4 months) metastasized to liver. (*H*) Amylase expression was absent in pancreatic cancer cells in cLGL-KRas/Ela-CreERT mice (*arrows*).